

Molecular cloning of flavonoid biosynthetic genes and biochemical characterization of anthocyanin *O*-methyltransferase of *Nemophila menziesii* Hook. and Arn.

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Received November 2, 2017; accepted January 4, 2018 (Edited by M. Mizutani)

Abstract Blue flower color of *Nemophila menziesii* Hook. and Arn. is derived from a metalloanthocyanin, nemophilin, which comprises petunidin-3-*O*-[6-*O*-(*trans-p*-coumaroyl)- β -glucoside]-5-*O*-[6-*O*-(malonyl)- β -glucoside], apigenin-7-*O*- β -glucoside-4'-*O*-(6-*O*-malonyl)- β -glucoside, and Mg²⁺ and Fe³⁺ ions. The flavonoid biosynthetic pathway of nemophilin has not yet been characterized. RNA-Seq analysis of the petals yielded 61,491 contigs. These were searched using BLAST against petunia or torenia flavonoid biosynthetic proteins, which identified 11 putative full-length protein sequences belonging to the flavonoid biosynthetic pathway. RT-PCR using primers designed on the basis of these sequences yielded 14 sequences. Spatio-temporal transcriptome analysis indicated that genes involved in the early part of the pathway are strongly expressed during early-petal development and that those in the late part at late-flower opening stages, but they are rarely expressed in leaves. Flavanone 3-hydroxylase and flavonoid 3',5'-hydroxylase cDNAs were successfully expressed in yeast to confirm their activities. Recombinant anthocyanin *O*-methyltransferase cDNA (*NmAMT6*) produced using *Escherichia coli* was subjected to biochemical characterization. *K_m* of *NmAMT6* toward delphinidin 3-*O*-glucoside was 22 μ M, which is comparable with *K_m* values of anthocyanin *O*-methyltransferases from other plants. With delphinidin 3-*O*-glucoside as substrate, *NmAMT6* almost exclusively yielded petunidin 3-*O*-glucoside rather than malvidin 3-*O*-glucoside. This specificity is consistent with the anthocyanin composition of *Nemophila* petals.

Key words: anthocyanin, flavonoid, flower color, methyltransferase, *Nemophila menziesii*.

Introduction

Flower color is one of the most important characteristics of floricultural crops. Red-to-blue flower color is derived from anthocyanins, a colored class of flavonoids. The flavonoid biosynthetic pathway, which is initiated by chalcone synthase (CHS; Figure 1) has been well characterized in terms of molecular biology, biochemistry, evolution, and interaction with pollinators (Grotewold 2006; Rausher 2006; Tanaka et al. 2008). The pathway leading to the biosynthesis of anthocyanidins and flavonol/flavone aglycones are well conserved among flowering plant species. Aglycones are modified with glycosyl and acyl moieties in a species-specific manner, which are then sequestered into the vacuoles

by glutathione *S*-transferase (GST)/ATP-binding cassette transporter, multidrug and toxic compound extrusion, and/or vesicle transport (Zhao and Dixon 2010). In some plant species, anthocyanins are further modified via glycosylation and acylation by vacuolar enzymes (Sasaki and Nakayama 2015). Anthocyanin structures greatly affect flower color. Most violet/blue flowers contain anthocyanins comprising delphinidin or its derivatives, petunidin, and malvidin as chromophores. Anthocyanin modifications with multiple aromatic acyl groups, presence of co-pigments (typically flavones and flavonols), metal ions (Fe³⁺ and Al³⁺), and higher vacuolar pH increase the intensity of blue in flowers (Yoshida et al. 2009).

Genes encoding enzymes or proteins involved in

Abbreviations: AMT, *S*-adenosylmethionine anthocyanin *O*-methyltransferase; ANS, anthocyanidin synthase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; EFP, enhancer of flavonoid production; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; GST, glutathione *S*-transferase; GT, glucosyltransferases.

This article can be found at <http://www.jspcmb.jp/>

Published online March 28, 2018

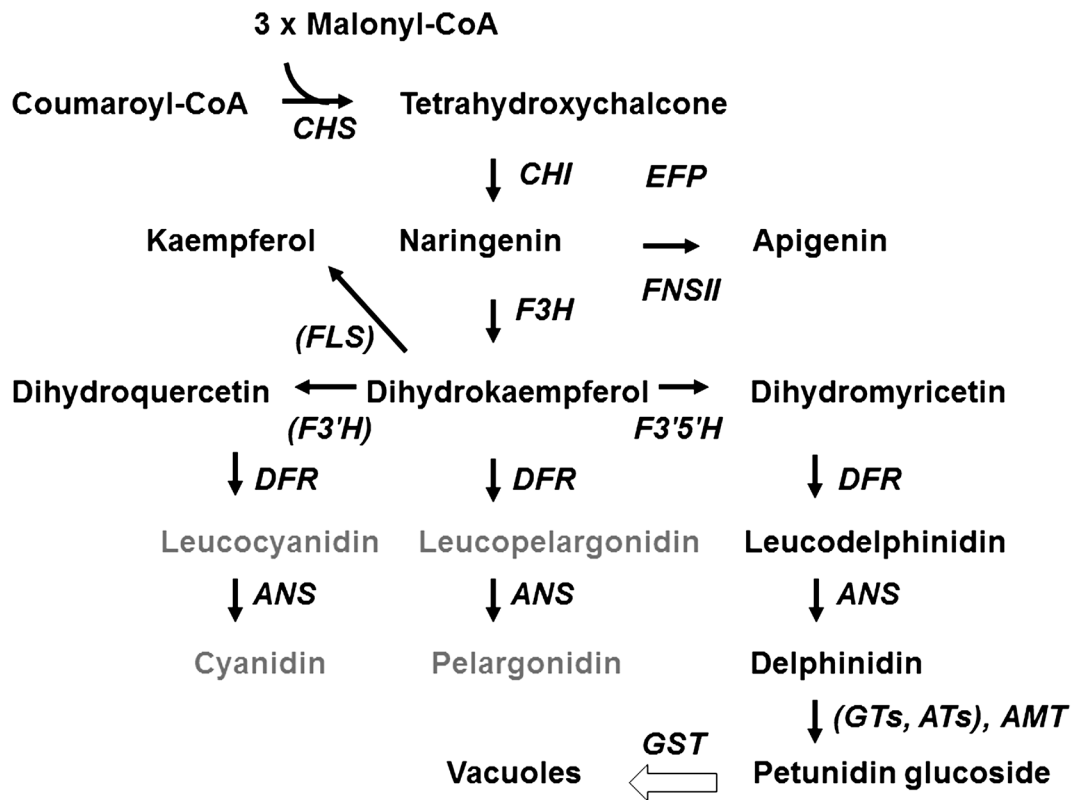


Figure 1. Schematic of a simplified flavonoid biosynthetic pathway in *N. menziesii*. Genes represented in parenthesis, including flavonol synthase (FLS), flavonoid 3'-hydroxylase (F3'H), glucosyltransferases (GT), and acyltransferases (AT), are not studied here. CHS, chalcone synthase; CHI, chalcone isomerase; EFP, enhancer of flavonoid production; FLS, flavonol synthase; FNSII, flavone synthase II; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; glucosyltransferases, (GTs); acyltransferases, (ATs); DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; AMT, S-adenosylmethionine anthocyanin O-methyltransferase; GST, glutathione S-transferase.

flavonoid biosynthesis have been isolated from many species. *Petunia* is a model species to study flower color and flavonoid biosynthesis (Koes et al. 2005). *Torenia*, an ornamental plant, has also been used as a model species to study flower color and shape though genetic modifications (Aida 2009). Notably, the development of DNA-sequencing technologies, such as transcriptome analysis, has enabled the isolation of flavonoid biosynthetic genes from many species, such as water lily (Wu et al. 2016) and *Dendrobium* (Li et al. 2017). Because most flavonoid genes appeared before the divergence of flowering plants (Rausher 2006), the function of isolated genes can usually be predicted on the basis of gene sequence and phylogenetic analyses. However, convergent evolution has been reported in case of two genes in this pathway, including flavone synthase I (FNSI), which evolved from *flavanone 3-hydroxylase* (F3H) gene in parsley (Martens et al. 2003), and *flavonoid 3',5'-hydroxylase* (F3'5'H) gene which independently evolved four times from *flavonoid 3'-hydroxylase* (F3'H) gene (Seitz et al. 2015). Therefore, functional analysis of F3H/FNSI and F3'H/F3'5'H homologues is necessary to identify their functions.

Due to genetic constraints, it is rare for a single plant species to bear flowers of all colors. F3'5'H is the

key enzyme in delphinidin biosynthesis (Tanaka and Brugliera 2013). Many important cut flowers, such as roses, carnations, and chrysanthemums, do not produce blue or violet flowers mainly because they do not accumulate delphinidin-based anthocyanins because of the genetic deficiency of *F3'5'H* gene. Expression of *F3'5'H* gene in these flowers successfully generated novel violet flowers, which hybridization breeding could not achieve (Brugliera et al. 2013; Katsumoto et al. 2007; Noda et al. 2013; Tanaka and Brugliera 2013). Transgenic carnations and roses have been commercialized. The accumulation of delphinidin 3-O-(6-O-malonyl) glucoside and delphinidin 3-O-(3,6-O-dimalonyl)-glucoside-3',5'-O-diglucoside in chrysanthemums through the introduction of *campanula F3'5'H* and butterfly pea *anthocyanin 3',5'-glucosyltransferase* genes resulted in true blue flowers, owing to the strong endogenous flavone co-pigments, such as luteolin 7-O-(6-O-malonyl)glucoside and apigenin 7-O-(6-O-malonyl) glucoside (Noda et al. 2017).

Nemophila menziesii Hook. and Arn., commonly known as "baby blue eyes," is a Boraginaceae plant native to western North America. *Nemophila* is utilized as a garden and pot plant to exhibit brilliant blue flowers. The color results from a metalloanthocyanin complex,

nemophilin, which consists of six molecules each of petunidin-3-*O*-[6-*O*-(*trans-p*-coumaroyl)- β -glucoside-5-*O*-[6-*O*-(malonyl)- β -glucoside] and apigenin-7-*O*- β -glucoside-4'-*O*-(6-*O*-malonyl)- β -glucoside, and one each of Mg²⁺ and Fe³⁺ ions (Yoshida et al. 2009, 2015). The *Nemophila* cultivar "Insignis Blue," which produces blue flowers, has been shown to contain both *cis* and *trans* forms of the anthocyanins, apigenin 7, 4'-*O*-diglucoside, apigenin 7-*O*-glucoside-4'-*O*-(6-*O*-malonyl)-glucoside, kaempferol 3-*O*-(6-*rhamnosyl*)-*O*-glucoside-7-*O*-glucoside, and kaempferol 3-(2,6-*O*-dirhamnosyl)-*O*-glucoside. Notably, its purple variant does not contain flavones (Tatsuzawa et al. 2014).

In spite of the eye-catching blue of *Nemophila* flowers, the molecular and biochemical aspects of its flavonoid biosynthetic pathway have not yet been investigated. In order to elucidate the biosynthesis of color constituents of *Nemophila* flower and to isolate possible useful molecular tools to engineer blue flower color, 14 genes encoding 11 flavonoid biosynthetic proteins were isolated using petal transcriptome data. Among these, functions of F3H and F3'5'H were confirmed in yeast. It is interesting that petunidin rather than malvidin is accumulated in the *Nemophila* petals, which prompted us to study AMT biochemically. AMT expressed in *Escherichia coli* was shown to selectively catalyze the biosynthesis of petunidin glucosides rather than malvidin glucosides. This result was consistent with the anthocyanin composition of *Nemophila* petals.

Materials and methods

Plant material

N. menziesii cultivar "Insignis Blue" was purchased from a florist and grown outside in Osaka, Japan. Flowers were classified into four stages (Figure 2A), and their petals were collected.

Flavonoid analysis

The collected petals of the four stages of flowers and leaves were lyophilized and their flavonoids were extracted using eight times (v/w) of 50% acetonitrile (v/v) containing 0.1% trifluoroacetic acid (TFA).

For anthocyanidin analysis, petal and leaf extracts (0.2 ml) were dried, dissolved in 0.2 ml of 6N HCl, and acid hydrolyzed at 100°C for 20 min to yield anthocyanidins. Anthocyanidins were extracted with 0.2 ml 1-pentanol and subjected to HPLC on an ODS-A312 column (15 cm×6 mm; YMC, Kyoto, Japan) at a flow rate of 1 ml min⁻¹ using an isocratic solvent (acetic acid:methanol:H₂O=6:7:27, v/v/v). Anthocyanidins were detected at an absorbance of 400–600 nm using a photodiode array detector (SPD-M20A, Shimadzu, Kyoto, Japan). For flavone and flavonol aglycone analysis, the dried petal and leaf extracts (0.2 ml) were enzymatically hydrolyzed in 0.2 ml of 0.1 M potassium phosphate buffer (pH 4.5) containing

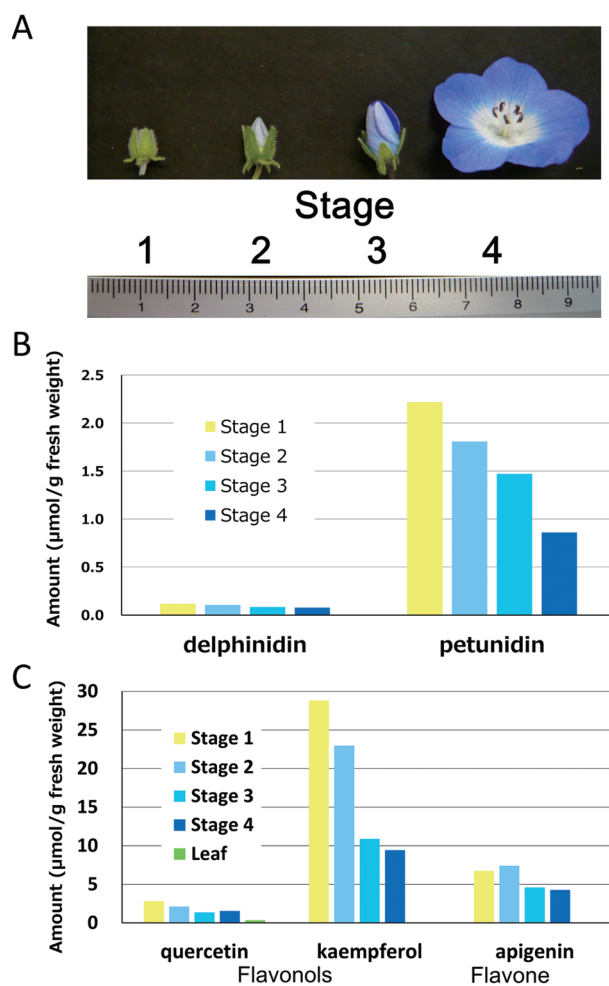


Figure 2. Developmental flavonoid analysis of *N. menziesii* flowers. A. Four developmental stages of *N. menziesii* flowers. B. Anthocyanidins at the four stages of the petals and leaves. C. Flavonol and flavone aglycones at the four stages of the petals and leaves.

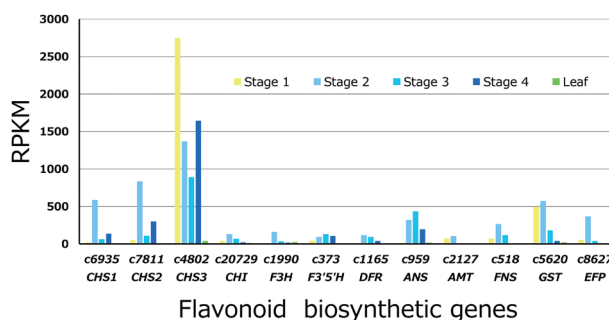


Figure 3. RNA-Seq analysis of flavonoid biosynthetic genes in *Nemophila*. The genes analyzed and their respective contigs are indicated on the X-axis, while the RPKM values per contig are indicated along the Y-axis.

6 units of β -glucosidase (Sigma, St. Louis, USA) and 1 unit of naringinase (Sigma) for 16 h at 30°C. The reaction was terminated by adding 0.2 ml of 90% (v/v) acetonitrile in water containing 0.1% TFA, and subjected to HPLC analysis using a Shim-pack FC-ODS column (15 cm×4.6 mm;

Shimadzu) with solvents A (H₂O:TFA=99.9:0.1, v/v) and B (H₂O:acetonitrile:TFA=9.9:90:0.1, v/v). The elution consisted of 20% solvent B to 100% solvent B for 10 min, followed by isocratic elution using 100% solvent B for 6 min at a flow rate of 0.6 ml min⁻¹. Flavonoids were monitored by absorbance at 250–450 nm using SPD-M20A photodiode array detector (SPD-M20A, Shimadzu).

Transcriptome analysis

Total RNA was isolated from the petals at all four stages and from leaves using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Total RNA from petals was used for transcriptome analysis (Eurofingentomics, Erlangen, Germany). Briefly, a normalized cDNA library was constructed from total RNA and sequenced using GS-FLX system (Roche, Basel, Switzerland). This generated approximately half a million reads with an average length of 400 bases, which were aligned to generate contigs with using Burrows-Wheeler Alignment tool (BWA) (Li and Durbin 2009).

Total RNA isolated from leaves and petals at four stages of

were subjected to RNA-Seq analysis to obtain transcript profiles (Eurofingentomics). A total of 120 million reads were obtained using HiSeq 2000. The number of reads were mapped on the alignments with using Sequence Alignment/Map tool software package (Li et al. 2009)

Flavonoid biosynthetic gene isolation

Generated sequences were searched with tBlastX using amino acid sequences of petunia or torenia flavonoid biosynthetic proteins [CHS, chalcone isomerase (CHI), enhancer of flavonoid production (EFP), FNSII, F3H, F3'H, F3'5'H, flavonol synthase (FLS), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), AMT, and GST; Figure 1] to obtain homologous contigs. The resulting sequences were aligned with their homologs from various plant species using CLUSTALW (DDBJ version; <http://clustalw.ddbj.nig.ac.jp/>, version 2.1, amino acid sequence alignment, default parameters), and a phylogenetic tree was constructed using Dendroscope 3 (Huson and Scornavacca 2012). Sequence reads for leaves and petals were mapped to contigs and used to

Table 1. List of flavonoid-related genes isolated in this study.

Gene name	Contig Number	Forward primer/Reverse primer	Accession Number
	Clone name		
Chalcone synthase	c6935 <i>NmCHS1</i>	TCTACGATCATGGAAGAATA/Oligo dT	LC328814
	c7811 <i>NmCHS2</i>	TCTACGATCATGGAAGAATT/Oligo dT	LC328815
	c4802 <i>NmCHS3</i>	CCGGCCCGAGAATGGTTAGC/Oligo dT	LC328816
Chalcone isomerase	c20729 <i>NmCHI1</i>	TATTCACAACAATGTCTACC/Oligo dT	LC328817
Enhancer of flavonoid production	c8627 <i>NmEFP3</i>	CAAGAAGATGTCTAGTGAAGTTG/ATCATTGGATAATTCAGCCAGAG	LC328824
	c8627 <i>NmEFP4</i>		LC328825
Flavone synthase	c518 <i>NmFNS2</i>	TACTAACATGGAGCTTTCG/Oligo dT	LC328818
Flavanone 3-hydroxylase	c1990 <i>NmF3H11</i>	AACCCCATGGCAACATTAAC/Oligo dT	LC328819
Flavonoid 3',5'-hydroxylase	c373 <i>NmF3'5'H10</i>	TTATACCCAATGGCCATGGC/Oligo dT	LC328820
Dihydroflavonol 4-reductase	c1165 <i>NmDFR1</i>	ACCAAGTATGACCACAATTG/Oligo dT	LC328821
Anthocyanidin synthase	c959 <i>NmANS1</i>	CAAACACCTCTAAATTCACC/Oligo dT	LC328822
Glutathione S-transferase	c5620 <i>NmGST</i>	AACATGGTGGTTAAAGTTTATGG/AAGAGATTGGATGAGTTGTGACG	LC328826
Anthocyanin O-methyltransferase	c2127 <i>NmAMT3</i>	CTCGAGATGTCTCTAGGACAAAC/ GGATCCTTAATAAAGACNCCGACATAG	LC330945
	c2127 <i>NmAMT6</i>		LC328823

calculate RPKM values for each contig.

Full-length cDNAs were obtained by RT-PCR using oligonucleotides containing a putative initiation codon and oligo dT primer or oligonucleotides containing a 3'-sequence of a stop codon of each contig (Table 1). RT-PCR was conducted using total petal RNA as the template and PrimeSTAR Max DNA Polymerase (Takara Bio, Otsu, Japan). The PCR parameters are as follows: denaturation at 98°C for 10 s, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 30 s, followed by a final extension at 72°C for 30 s and subsequent cooling to 4°C. The amplified DNA fragments were cloned into pCR-TOPO vector (Life Technologies, Carlsbad, CA) and sequenced.

Functional expression analysis *in vitro*

A yeast expression vector, pYES2 (Life Technologies, Carlsbad, CA), was used to express *NmF3H* and *NmF3'5'H* cDNAs following manufacturer's protocol. Crude extracts of yeast transformants were subjected to 2-oxoglutarate-dependent dioxygenase assay for F3H (Saito et al. 1999) and cytochrome P450 monooxygenase assay for F3'5'H (Ueyama et al. 2002) using naringenin as the substrate. The reaction mixtures were extracted with ethyl acetate, dried, and subjected to HPLC analysis using Shim-pack FC-ODS column (150 mm × 4.6 mm; Shimadzu) at 40°C and a flow rate of 0.6 ml/min. The elution using solvents A and C (H₂O:acetonitrile:TFA=49.9:50:0.1, v/v) consisted of a 10 min linear gradient from 80% solvent A and 20% solvent C to 70% solvent C, 6 min isocratic elution with 70% solvent C, 1 min linear gradient to 20% solvent C, and 11 min of 20% solvent C. Flavonoids were detected at an absorbance of 280 nm using a photodiode array detector (SPD-M20A, Shimadzu).

cDNAs of *NmAMT* and petunia AMT encoded by the *MT2* locus (GenBank/EMBL/DDBJ database accession number: KJ676515.1, Provenzano et al. 2014) were sub-cloned into pET15b expression vector (Novagen, Darmstadt, Germany) and expressed in *E. coli* using the Overnight Express Autoinduction System 1 (Novagen, Darmstadt, Germany) at 18°C for two overnights. Recombinant proteins were purified using His-Tag affinity chromatography with Profinia protein purification system (Bio-Rad, Hercules, CA). Purified proteins were quantified using the Bio-Rad protein assay. AMT assay mixture (0.1 ml) comprised 40 mM KPi (pH 7.5), 50 μM anthocyanin, 1 mM S-adenosylmethionine, 2 mM MgCl₂, and 3 μg recombinant protein. The mixture was incubated at 30°C for 15 min, and the reaction was terminated by adding 90% acetonitrile containing 0.1% TFA and 0.24 N HCl. The reacted mixture was then subjected to HPLC analysis using a Shodex RSpak DE-413L column (250 mm × 4.6 mm, Showa Denko, Tokyo, Japan) at 40°C and a flow rate of 0.6 ml/min. The sample was eluted using solvents D (H₂O:TFA=99.5:0.5, v/v) and E (H₂O:acetonitrile:TFA=49.5:50:0.5, v/v/v), and the elution consisted of 15 min linear gradient from 20% solvent E to 100% solvent E, 10 min isocratic elution with 100% solvent B, and 1 min linear gradient to 20% solvent E. Anthocyanins were

detected at an absorbance of 280 nm using a photodiode array detector (SPD-M20A, Shimadzu).

K_m value for delphinidin 3-O-glucoside was determined by 12 min reaction with the substrate at concentrations ranging from 5–150 μM using the Lineweaver–Burk plot. This experiment was repeated three times.

Results and discussion

Flavonoid analysis of *Nemophila* petals

The results of flavonoid analysis are summarized in Figure 2B and 2C. Cyanidin and pelargonidin are not detected. Small amounts of peonidin and malvidin were detected. No anthocyanidins were detected in leaves. The petunidin concentration at stage 4 was 0.86 μmol/g fresh weight. Myricetin and tricetin were not detected. The stage 4 petals contain 1.54 μmol/g quercetin, 9.45 μmol/g kaempferol, and 4.28 μmol/g apigenin. Leaves contained 0.36 μmol/g quercetin and 0.03 μmol/g kaempferol. Concentrations of anthocyanins, flavonols, and flavones in petals decreased as petals developed, whereas leaves rarely contained flavonoids. Biosynthesis of flavonols precedes that of anthocyanins in petals of most plants, such as petunia (Holton et al. 1993) and rose (Tanaka et al. 2003). However, this trend was unclear in *Nemophila*.

Apigenin was the almost exclusive flavone. Although nemophilin comprised petunidin and apigenin glucosides in equal molar ratios, the amount of apigenin was about 2–3 times higher than that of anthocyanins. More importantly, petals contain a greater amount of flavonols than flavones and anthocyanins. Functions of flavonols in *Nemophila* petals have not yet been elucidated. It is possible that flavonols and excess amount of flavones function to attract pollinators because they absorb UV light or have other important biological functions.

Expression and phylogenetic analysis of flavonoid-related genes

A total of 61,491 contigs was obtained and subjected to search of flavonoid biosynthetic genes. The contig sequences of the flavonoid biosynthetic genes are summarized in Table 1. Three CHS homologue sequences were isolated and two of them, *NmCHS1* (contig 6935) and *NmCHS2* (contig 7811), were very similar. These showed only five residue differences in 386 amino acid residues (98.4% and 98.4% identities in amino acid and nucleotide sequences, respectively). Another CHS homologue, *NmCHS3* (contig 4802), showed significant differences from *NmCHS1* (89.1% and 83.3%, respectively) and *NmCHS2* (90.4% and 83.4%, respectively). CHS homologs have been isolated from a number of plants. The genus *Nemophila* belongs to the family Boraginaceae, which was previously a part of the order Lamiales; however, it is now classified under

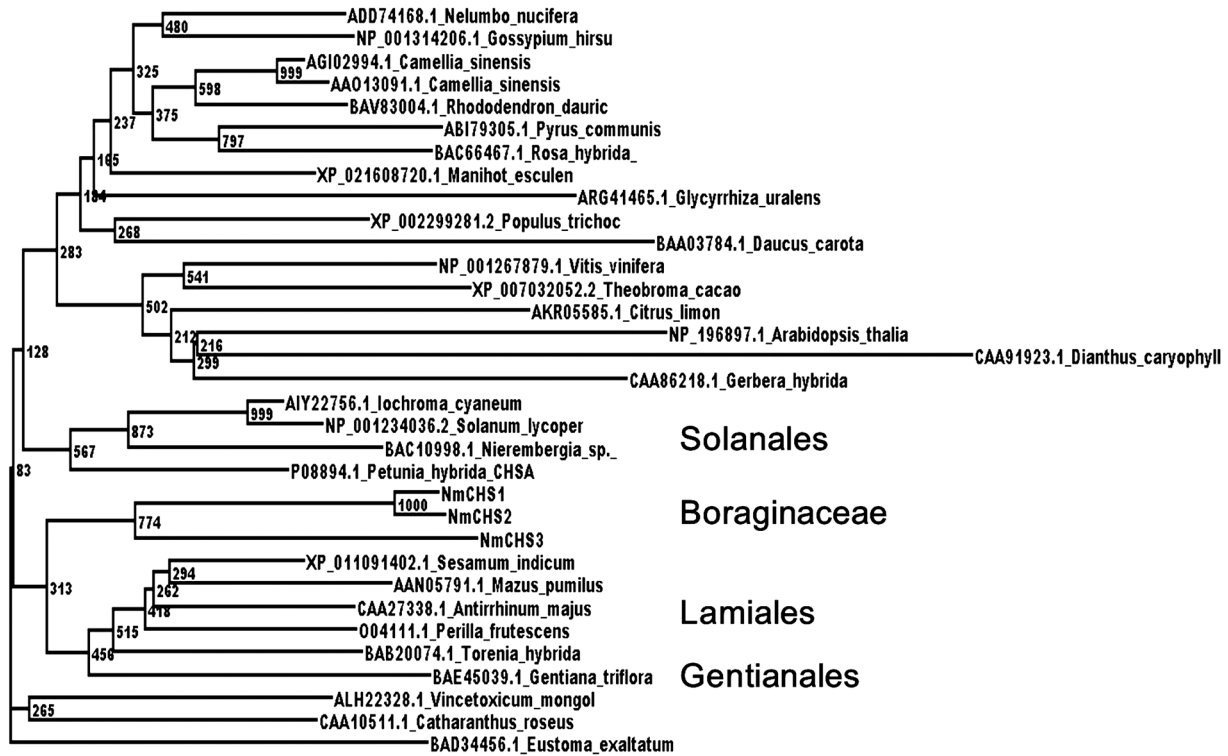


Figure 4. Phylogenetic cluster analysis of chalcone synthases genes across plant species. Accession numbers in the database and species names are shown. Bootstrap values out of 1000 replicates are shown.

order Boraginales (APG IV 2016). The phylogenetic analysis of CHS indicates that *Nemophila* CHS is related to CHSs of plants belonging to orders Lamiales, Gentianales, and Solanales, but it is not included in the clades containing these plant orders (Figure 4).

Two cDNAs per contig were isolated for *EFP* (*NmEFP3* and *NmEFP4*, 98.1% and 97.0% identities in amino acid and nucleotide sequences, respectively) and *AMT* (*NmAMT3* and *NmAMT6*, 97.8% and 97.9% identities, respectively) genes. Although the molecular mechanism of *EFP* function is unknown, it has been recently reported to be involved in flavonoid biosynthetic pathway (Morita et al. 2014). *Nemophila* data also suggest the involvement of *NmEFP* in the flavonoid biosynthetic pathway. *F3'H* counterparts were not found in the *Nemophila* transcriptome, although the presence of quercetin in petals (Figure 2C) suggests the presence of *F3'H* gene in the *Nemophila* genome. Only partial sequences of *FLS* were found, which was not further studied in *Nemophila*.

Expression profiles of isolated genes on the basis of RPKM values are shown in Figure 3. *CHS3* expression was most abundant at stage 1, and it was more abundant than *CHS1* and *CHS2* expressions suggesting that it may play a major role in flavonoid biosynthesis. *CHI*, *EFP*, and *F3H* expressions were the highest at stage 2, whereas *F3'5'H* and *ANS* expressions were strongest at stage 3. Flavonoid biosynthetic genes have been divided into two groups designated as “early” and “late” (Martin et al.

1991; Quattrocchio et al. 1993), which is also applicable to *Nemophila* flavonoid pathway. Interestingly, *AMT*, which functions at later stages than *ANS* significantly expressed only at stages 1 and 2.

Functional analysis of *NmF3H*, *NmF3'5'H*, and *NmAMT*

The flavonoid biosynthetic pathway involves several 2-oxoglutarate dioxygenases (*F3H*, *ANS*, *FLS*, and *FNSI*) and cytochrome P450 monooxygenases (*F3'H*, *F3'5'H*, and *FNSII*). *NmF3H11* and *NmF3'5'H10* genes were expressed in yeast and their activities were successfully measured in this study (Supplemental Data). Dihydrokaempferol was generated from naringenin when yeast extract containing recombinant *NmF3H11* was subjected to the assay. Yeast expressing *NmF3'5'H10* produced eriodictyol and pentahydroxyflavanone from naringenin. *NmF3'5'H10* catalyzed the hydroxylation of naringenin more efficiently than petunia *Hfl F3'5'H* expressed under the same conditions (Data not shown). *NmF3'5'H10* can be a useful molecular tool to shift the flavonoid biosynthetic pathway to delphinidin biosynthesis by which flower color can be modified to blue hue (Tanaka and Brugliera 2013).

Since *Nemophila* dominantly accumulates petunidin type anthocyanins rather than malvidin type anthocyanins, substrate specificity of *Nemophila* *AMT* was elucidated. Among the two *AMT* homologues (*NmAMT3* and *NmAMT6*), *NmAMT6* was subjected

Table 2. Comparison of substrate preference of NmAMT6 and petunia MT2 AMT.

Substrate	Relative activities (%)	
	NmAMT6	petunia MT2 AMT
Delphinidin 3- <i>O</i> -glucoside	100% (Pet 97.8%, Mal 2.2%)	100% (Pet 90.3%, Mal 9.7%)
Delphinidin 3,5- <i>O</i> -diglucoside	85.7% (Pet 86.0%, Mal 13.0%)	46.9% (Pet 98.8%, Mal 1.2%)
Petunidin 3- <i>O</i> -glucoside	8.75%	25.0%

Relative activities are shown relative to delphinidin 3-glucoside, which was set at 100%. Data represents averages of three independent assays. Percentages of petunidin (Pet) and malvidin (Mal) glucoside are shown in parentheses.

to biochemical analysis because they differ only 5 amino acid residues out of 232 residues and preliminary enzymatic characterization of the recombinant NmAMT3 and NmAMT6 indicated that their substrate preferences are similar (data not shown). Comparative analysis of substrate and product specificity of NmAMT6 and petunia MT2 AMT was conducted (Table 2). Petunia MT2 AMT preferably produces petunidin over malvidin (Provenzano et al. 2014). The methylation efficiency of recombinant NmAMT6 was much higher for the substrates delphinidin 3-*O*-glucoside and delphinidin 3,5-*O*-diglucoside than for petunidin 3-*O*-glucoside. In contrast, petunia MT2 AMT preferably catalyzed methylation of delphinidin 3-*O*-glucoside than of delphinidin 3,5-*O*-diglucoside and petunidin 3-*O*-glucoside. Reactions of the recombinant NmAMT6 with delphinidin 3-*O*-glucoside and delphinidin 3,5-*O*-diglucoside produced petunidin 3-*O*-glucoside and petunidin 3,5-*O*-diglucoside and only small amounts of malvidin 3-*O*-glucoside (2.2% of total products) and malvidin 3,5-*O*-diglucoside (13.0% of total products), respectively. Petunia MT2 AMT yielded more malvidin 3-*O*-glucoside (9.7%) from delphinidin 3-*O*-glucoside than NmAMT6 and less malvidin 3,5-*O*-diglucoside (1.2%) from delphinidin 3,5-*O*-diglucoside. Interestingly, the ratio of malvidin glucosides produced varied depending on substrates. The low production of malvidin glucosides by NmAMT6 and petunia MT2 AMT is in contrast to that by grape (Hugueney et al. 2009), petunia MF1 and MF2 (Provenzano et al. 2014), and torenia (Nakamura et al. 2015) AMTs, which were demonstrated to efficiently catalyze 3' and 5' methylations of delphinidin glucosides to yield malvidin glucosides. Cyclamen AMT was also shown to catalyze malvidin production (Akita et al. 2011). The low production of malvidin glucosides by NmAMT6 (Table 2) is possibly due to its low reactivity to petunidin glucosides. This specificity explains the anthocyanidin composition of *Nemophila* petals containing petunidin but not malvidin. Because NmAMT6 catalyzes the methylation of both delphinidin 3-*O*-glucoside and delphinidin 3,5-*O*-diglucoside, *Nemophila* anthocyanin biosynthetic pathway may form a metabolic grid as reported for many other plants, such as perilla (Gong et al. 1997) and gentian (Fukuchi-Mizutani et al. 2003).

Depleting Mg²⁺ from the reaction mixture decreased

the activity of NmAMT6 to <2% (Data not shown), indicating that NmAMT6 activity depends on Mg²⁺, as is true for AMTs of other plants, including grape (Hugueney et al. 2009). *K_m* and *k_{cat}* values for delphinidin 3-*O*-glucoside of NmAMT6 were 26.8±4.67 μM and 13,100±312 s⁻¹, respectively. The *K_m* value of NmAMT6 is consistent with that of torenia (Nakamura et al. 2015), grape (Hugueney et al. 2009), tomato (Roldan et al. 2014), and peony (Du et al. 2015) for anthocyanins ranging from 1.1–123 μM.

This study confirms that flavonoid/anthocyanin-related genes could be comprehensively isolated by transcriptome analysis of petals. The genes isolated from *Nemophila* could be classified into early and late genes and these genes were generally expressed in a well-coordinated manner as reported in many other plants. Additionally, the product specificity of NmAMT6 was consistent with the anthocyanidin composition of *Nemophila* petals.

Acknowledgements

The authors thank Ms. Ishiguro, Ms. Ohnishi, Ms. Takeuchi for their technical assistance. We also thank our colleagues for their help and encouragement.

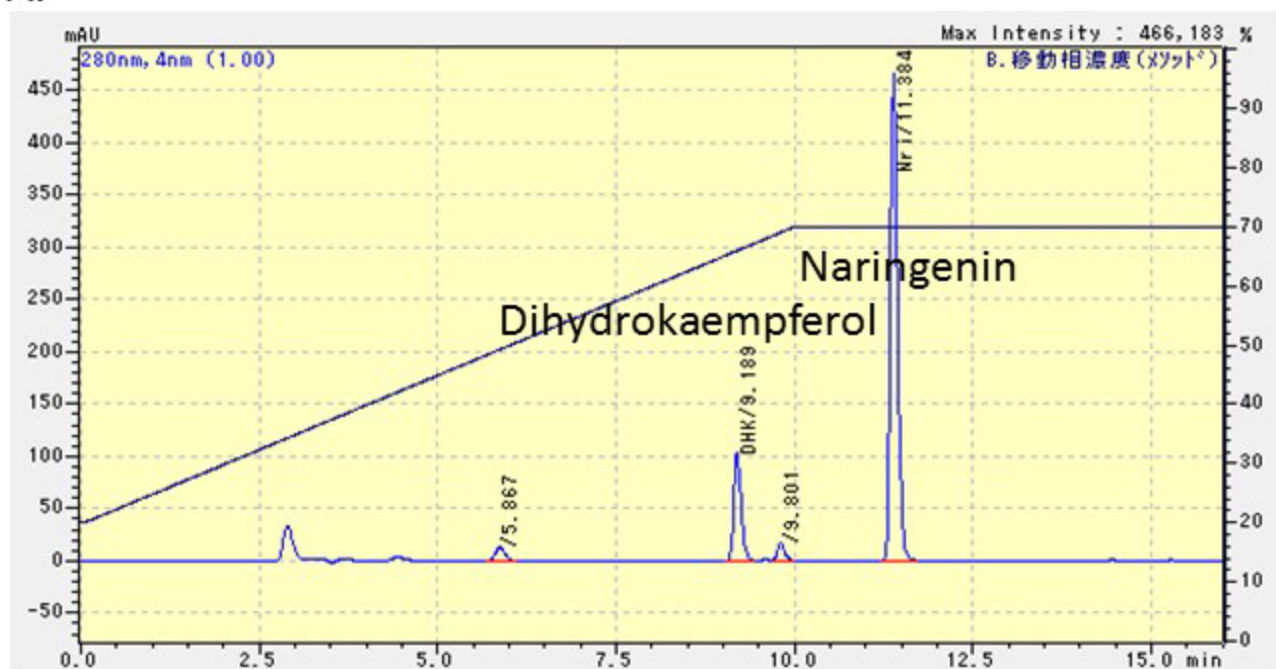
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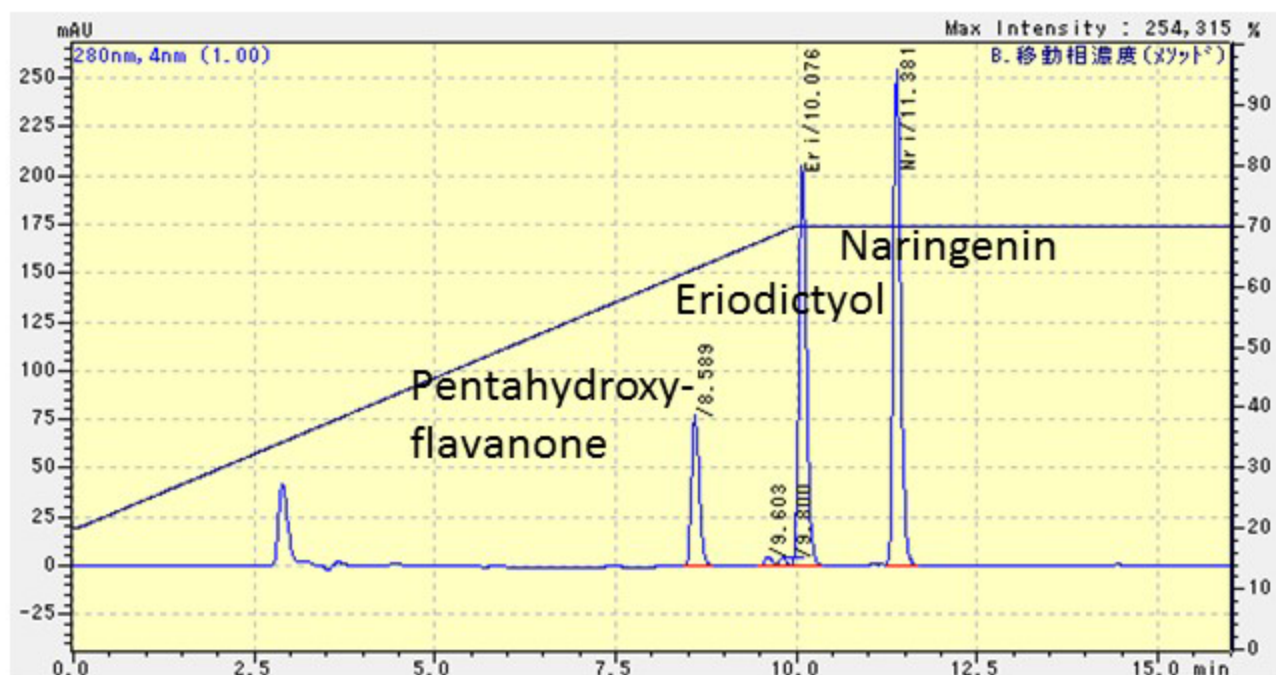
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Supplemental data

A.



B.



HPLC profiles of flavanone 3-hydroxylase (A) and flavonoid 3',5'-hydroxylase (B) assay using protein crude extracts of yeast expressing NmF3H11 and NmF3'5'H10, respectively.